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Laboratory Investigation of Childhood Enteric Infections

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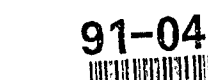
Introduction

This chapter will review current *diagnostic methods* for a variety of enteropathogens including bacteria, viruses and parasites. These methods may include: (1) direct observation in stool; (2) culture and isolation from stool or biopsy specimens, often through the use of selective or enrichment media; (3) antibody-enhanced detection of organisms or their products in stool and identification after culture; (4) identification of specific material in stool or culture; (5) determination of host serum or secretory immune responses to the organisms or their products.

Major advances in diagnostic methods have come in the areas of: (1) improved methods for selective culture or isolation of "newly recognized" pathogens; (2) greater commercial availability of specific high titer antibodies particularly specific monoclonal antibodies, for novel, rapid diagnostic tests; (3) application of the techniques of molecular biology, in particular genetic analysis and the development of specific genetic probes for the identification of organisms or their virulence determinants.

In this chapter we will discuss *enteropathogens of major importance* in the categories of bacteria, viruses and protozoal parasites. For each pathogen we will briefly review clinical presentation, epidemiolgy and current taxonomy; we will then review current procedures for sample

[1] The views expressed herein are those of the authors and not necessarily those of the United States Army or the Department of Defense



collection, transport, isolation and identification of organisms with emphasis on new techniques. Where applicable we will also discuss serodiagnosis. Finally, we will summarize our current recommendations for approach to diagnosis.

The expanding application of *molecular biology techniques* to the identification of enteric pathogens deserves special attention at the beginning of this chapter on newer methods for diagnosis. The techniques of plasmid profile analysis, restriction endonuclease analysis of plasmid and chromosomal DNA, and nucleic acid hybridization with specific DNA probes are finding increasing application in epidemiologic studies, and, to a lesser extent, in clinical microbiology [1, 2]. To date, these have been applied primarily to bacterial pathogens, and to a lesser extent to viruses.

Plasmid Analysis [2]

Since many of the virulence properties of bacterial enteropathogens are encoded on these extrachromosomal DNA elements, the analysis of plasmid patterns after electrophoresis in agarose gels and staining with ethidium bromide may provide a recognizable "finger print" of an organism. Plasmid preparations sufficient for analysis can be extracted from small volumes of overnight broth cultures. Bacteria are generally lysed with combined treatment with EDTA, lysozyme and detergent at an alkaline pH which irreversibly denatures chromosomal DNA. After neutralization, only plasmid DNA is present in a form that remains in the supernatant after brief centrifugation and is precipitated by ethanol in the cold. Electrophoretic analysis separates and displays plasmids by their molecular weight. This analysis may be made more specific by first digesting the plasmides with restriction endonucleases. These enzymes cut the plasmid DNA at specific nucleotide sequences, yielding smaller fragments with very characteristic profiles on electrophoresis.

Bacterial Restriction Endonuclease DNA Analysis (RRENDA)

Endonuclease digestion can also be applied to the analysis of total chromosomal DNA, since not all enteric pathogens have characteristic plasmids. DNA is again obtained from lysed cells and extracted with phenol and cholorform to eliminate contaminants. More complex, but equally characteristic, patterns are obtained on electrophoresis of digested chromosomal DNA than are seen with plasmid DNA [1].

Construction and Utilization of Genetic Probes

Genetic probes are labelled, well-characterized, single-stranded DNA fragments whose nucleotide sequences are unique to a particular pathogen. These probes can be used to

recognize homologous, complementary, DNA sequences in cultures or clinical stool specimens through DNA/DNA hybridization.

To obtain a probe for a virulence determinant encoded on a plasmid, the genetic material of interest must first be cloned [2]. To do this, the native plasmid is isolated, digested with a restriction endonuclease, and the resulting fragments religated into a plasmid cloning vector containing an antibiotic resistance marker (e.g. pBR322). The resulting recombinant plasmids are then used to transform a recipient strain (such as HB101). Transformed colonies are selected by antibiotic resistance, then screened for expression of the desired virulence determinant. Recombinant plasmids from colonies so identified are isolated and digested with the same restriction enzymes to identify the cloned fragments of interest. Fragments are separated on agarose gels, and electroeluted, before labelling with ^{32}P by nick translation with T4 polynucleotide kinase. Alternatively, if the nucleotide (or protein) sequence is known, oligonucleotide probes can be synthesized to conform to a known sequence and labelled. Oligonucleotide probes may be more specific since they will contain no vector DNA.

The ^{32}P-labelled DNA is used to probe the total DNA in clinical specimens, or in isolated colonies, by hybridization by a modification of the method of Grunstein and Hogness [3]. Stools samples, or colonies, are spotted onto nitrocellulose filters overlying nutrient agar and allowed to grow overnight. The filters are then treated with NaOH to lyse the bacteria and denature DNA. The DNA is fixed by baking overnight at 65 °C before probing. Preparation of the samples to this point can be performed in the field. Hybridization is performed in hybridization solution containing formamide, citrate, Ficoll PVP and BSA. Labelled probe DNA (10^5–10^6 cpm) is added in the presence of excess sheared carrier DNA for overnight incubation. Detergent washed filters are autoradiographed by exposure to X-ray film with an intensification screen for 24 h at −70 °C, so that positive colonies are identified as dense spots.

Since it takes three days to complete these procedures, hybridization techniques cannot be considered rapid diagnostic methods. Their utility lies in their ability to handle large numbers of samples reproducibly. As a result they are usually used to simultaneously investigate a large number of samples from epidemiological studies rather than being applied to diagnosis of individual cases of diarrhea. Use of stable isotopes or nonradioactive detection systems should increase the utility of these probe systems since ^{32}P-labelled probes have a finite half-life. Another limitation of gene probes is that they detect virulence determinants which are encoded but which may not be expressed, thus they may yield false-positive results for pathogenicity.

Salmonella

Salmonellae cause both diarrheal disease and systemic infection, but the most common manifestation of Salmonella infection, of whatever group, species, or serotype is gastroenetritis [4, 5]). Salmonella are classified into three major groups (I, II and III) according to the spectrum of clinical disease they usually produce [5]. At present in the United States, three species are recognized which correspond to groups I to III. Group I

organisms (species *S. enteritidis*) generally cause diarrheal disease (although they may cause severe systemic disease in infants, the elderly, and the immunocompromised). Group I includes all but 2 of the more than 2,000 serotypes which had been recognized by 1988, and which were classically named after the host, or location in which they were isolated (e.g. typhimurium, heidelberg, enteritidis, newport, infantis). Group II organisms (species *S. cholerasuis*) cause septicemias and are associated with localized suppurative infections in adults, but they often cause gastroenteritis in children. Group III (species *S. typhi*) organisms cause typhoid (enteric) fever in humans, but can also have an initial presentation as a diarrheal illness, especially in children [6]. Clinical features cannot be used to differentiate Salmonella diarrhea from that produced by other enteric pathogens. Isolation of the organism from stool or blood cultures is the only way to confirm the diagnosis. Most patients have mild crampy abdominal pain with watery to paste-like stools, but a dysentery-like illness with small-volume bloody stools may occur [7]. Salmonellae pathogenic for humans colonize the intestinal tracts of many animal species including swine, poultry and cattle. Outbreaks of gastroenteritis due to Salmonella often develop after the consumption of contaminated food, in particular eggs, dry milk, and chicken. A DNA probe, such as that which detects the Vi capsular antigen gene [8, 9], may eventually shorten the time required for diagnosis.

Procedures

Salmonella are present in the stool in appreciable numbers only during the acute stage (first 3 days) of diarrheal disease, so specimens for culture should be obtained during this period if at all possible. Because many commensals proliferate more rapidly than Salmonellae, specimens should also be inoculated into transport media such as (Carey-Blair, 0.033 *M* phosphate buffer mixed with equal parts of glycerol, Ames, or Stuart's) and then onto appropriate culture media as soon as possible. Salmonella are differentiated in culture from other Enterobacteriaceae by their failure to ferment lactose, ability to resist the inhibitory effect of citrate, and by producing hydrogen sulfide in triple sugar iron agar (TSI) [4]. Miniaturized multiple-test systems that incorporate multiple biochemical reactions are now in widespread use and have simplified and shortened the process of identification.

Once isolated, Salmonella can be *serotyped* using the slide agglutination method [10]. This is of great importance in epidemiological studies,

such as to identify a common source for an outbreak, but is less useful for the clinician. The large number of serotypes are grouped by the O, or somatic antigen, and typed by their H, or flagellar antigen. Because approximately 65 different somatic antigens have been recognized, subgrouping with polyvalent O antiserum is a necessity, and commercially prepared kits are available (Lederle Laboratories, Pearl River, N.Y.: Difco Laboratories, Detroit, Mich.; Baltimore Biological Laboratory, Cockeysville, Md.; Lee Laboratories, Grayson, Ga.). From a practical standpoint, the cultures to be tested should be smooth and must not autoagglutinate in saline [10]. Also if no agglutination occurs with the O diagnostic sera, the culture should be heated to 100 °C for 15–30 min, cooled, and retested, since some Salmonellae possess K antigens and are nonagglutinable in the live or encapsulated form in O antisera. O nonagglutinable cultures must be sent to reference laboratories for complete serologic analysis.

Isolation of organisms in pure culture and performance of biochemical and serological assays are cumbersome, time-consuming, and expensive processes. Techniques of genetic analysis from the field of molecular biology have begun to be applied to salmonella diagnosis.

An example of the use of plasmid profile analysis in the epidemiology of Salmonella is provided by Taylor et al. [11]. Marijuana was suspected to be the most likely common source of infection for an outbreak of *S. muenchen*, but this was difficult to prove, since isolates studied were phenotypically identical to other *S. muenchen* strains. The issue was clarified, however, by the demonstration of both a common plasmid profile and a common restriction digest pattern for the plasmids recovered from patients, and drug isolates. The reliability of restriction enzyme analysis as an epidemiologic tool was demonstrated, since the restriction patterns remained virtually unaltered despite enteric passages in many naturally infected individuals.

A recently constructed DNA probe was validated as highly effective at distinguishing *S. typhi* from other gram-negative bacteria and also other strains of Salmonella in subcultures of diarrheal specimens collected in Peru and Indonesia [8]. The viaB genetic locus of *S. typhi* is involved in the synthesis of the capsular virulence (Vi) antigen expressed by virulent *S. typhi* strains. The DNA probe used to detect this locus is an 8.6-kb EcoRI fragment from plasmid pWR141, which was constructed by cloning the Vi antigen locus of a *Citrobacter freundii* strain [9]. At present, this technique requires isolation of colonies from fresh specimens, and overnight culture on filters, to obtain sufficient concentrations of the bacterial DNA.

Recommendations

Diagnosis of salmonellosis requires isolation of the organism. Positive blood, bone marrow, or urine cultures are confirmatory. Positive stool

cultures may indicate enteric infection, but they may also indicate a carrier stat, presumably related to intermittent secretion of the organisms from the biliary tree. Culture is not cost-effective for patients with mild watery diarrhea and who are afebrile with only mild systemic symptoms, since the illness often resolves before culture results are known. Culture may be indicated, however, to identify outbreaks of Salmonellosis caused by common sources, particularly in nurseries or in nursing homes. If dysentery, fever, or severe symptoms are present, culture is necessary to guide therapy. Serology is not useful clinically. Techniques of plasmid analysis are currently useful for epidemiological studies. DNA probes currently available are reliable for identifying subcultured organisms, and may be applied to direct detection of *S. typhi* directly in stool or blood specimens if methods to concentrate bacterial DNA can be developed.

Shigella

Shigella are highly virulent, locally invasive organisms which are the major cause of dysentery [4, 12, 13]. Oral ingestion of as few as 100 organisms can result in the full clinical syndrome [14]. Stools are characteristically bloody and small in volume, with greater than 50 WBC/HPF on fecal smears. Nonintestinal manifestations may be severe, and include fever with convulsions, leukemoid reactions, hemolytic-uremic syndrome, and reactive arthritis. Watery diarrhea may also occur. The host range of Shigella species is limited to humans and primates [14]. Spread is from person-to-person or through fecal contamination of water supplies. The clinical importance of Shigellae stems from their unique virulence and also from their emerging antibiotic resistance. In the Asian subcontinent, drug-resistant *S. dysenteriae I* infections are currently achieving epidemic proportions. Four species (or groups) are identified on the basis of biochemical and serological reactions: *S. dysenteriae* (group A), *S. flexneri* (group B), *S. boydii* (group C), and *S. sonnei* (group D.) Diagnosis continues to depend on the identification ot the organisms in stool by culture. New developments in diagnosis include preparation of a DNA probe based on virulence determinants cloned from the 140-Mdalton virulence plasmid which confers invasive ability upon Shigellae and enteroinvasive *Escherichia coli* [15-17]. In addition, a high titer diagnostic antiserum, raised against surface antigens associated with virulence, has been utilized in an ELISA [18].

Procedures

Shigella are slender gram-negative nonmotile rod-shaped members of the Enterobacteriaceae family. The standard laboratory diagnosis of Shigella infection has changed little over recent years. Stool specimens are cultured on differential and selective media and a screening battery of biochemical tests are performed on isolates. Stool specimens for Shigella culture need to be obtained early in the course of the illness, and, if possible, should include bits of bloody mucus or epithelium which may contain invading organisms. Immediate culturing of material obtained during proctoscopic examination increases the likelihood of obtaining positive Shigella cultures [10, 19]; the organism quickly dies in acid stool specimens. By genetic studies, Shigella are extremely closely related to *E. coli* [13], although they are nonlactose fermenters. They are oxidase negative, facultatively anaerobic, and non-spore-forming. In distinction to Salmonella, Shigella do not produce hydrogen sulfide, but have a fermentation pattern similar to Salmonella on TSI agar. The overall scheme for serological identification of Shigella is similar to that for Salmonella. Because of the number of serotypes (group A, 10; group B, 6; group C, 15; group D, 1) subgrouping is done with polyvalent antisera, and commercial kits for slide agglutination tests are available (Lederle Laboratories; Difco Laboratories; Baltimore Biological Laboratory; Lee Laboratories).

The shared virulence attributes of Shigella and enteroinvasive *E. coli* include their ability to invade epithelial cells, to multiply intracellulary and to spread intercellularly to adjacent cells. The genetic determinants of these properties have been the subject of intense research interest and several in vivo and in vitro assays have been developed for their study. The production of keratoconjuctivitis in the eye of a guinea pig (Sereny test) [20] has been a standard confirmatory test for invasive ability, but is impractical for testing large numbers of strains. Other tests include the demonstration of HeLa cell invasiveness in tissue culture which relies on the ability of invading organisms to survive externally applied antibiotics. This test has been refined into a plaque assay which reflects the intercellular spread of the organisms [21]. These tests are not used for routine diagnosis, but have primarily been utilized to define the genetic and phenotypic determinants of Shigella virulence which include the products of both chromosomal and plasmid loci.

An additional research method to detect invasion-associated virulence factors but whose future importance in clinical diagnosis remains to be determined is an ELISA assay [18].

Invasive strains of Shigella or *E. coli* express a "virulence marker antigen" (VMA) on their surface, reflecting Sereny test positivity. The critical reagent for the ELISA is an absorbed antisera that detects the VMA. This was raised in rabbits by immunization with an invasive *E. coli* strain, then absorbed with an organism of the identical serotype, biotype, antibiotic susceptibility, and plasmid pattern, but which had been rendered Sereny negative by serial passage on nutrient agar. Microtiter wells are coated with test bacteria, then incubated with the anti-VMA antisera followed by an antirabbit IgG conjugate. Fifty-six Sereny test positive strains gave high OD values (range 0.4–1.6) and 27 Sereny test negative strains gave a low OD (range 0–0.15). With one exception, no VMA-positive strain harbored the 140-Mdalton plasmid [18]. This method requires that pure cultures of isolated organisms be used for sensitizing wells, and it has not been applicable to testing stool samples directly, making its use in diagnosis or for screening large numbers of isolates impractical.

Techniques of plasmid analysis have led to the discovery that large plasmids in the range of 140 Mdaltons (120 Mdaltons in the case of *S. sonnei*) confer most of the organism's invasive ability [15] and code for outer membrane proteins required in the recognition and penetration process [16, 17, 22]. High degrees of homology have been demonstrated for the invasive plasmids of the several Shigella species tested, perhaps reflecting their common origin [23]. The relative ease of plasmid detection and the firm evidence that large plasmids are required for invasion has made plasmid analysis an attractive alternative to other test for virulence. Detection of the invasion plasmid has been facilitated by application of DNA hybridization techniques. A 17-kb radiolabelled DNA probe, prepared from the 140-Mdalton virulence plasmid of *S. flexneri 5* has been shown to permit sensitive and specific screening of cultured strains for the invasive genotype, thereby permitting analysis of many more specimens than possible by conventional assay [24, 25]. Sensitivity in direct analysis of stool specimens directly is less than optimal, however. The clinical need to more rapidly identify pathogens in cultures has given impetus to the development of improved hybridization probes. Because radiolabelled probes decay rapidly and have a limited useful life, a biotinylated 17-kb DNA probe has been developed and shown to compare favorably with the autoradiographic assay in terms of sensitivity and specificity [26]. In one study, both radioactive and biotinylated probes hybridized with stool blots from 11 of 13 Thai children with culture-proven shigellosis or EIEC diarrhea. All 43 children with cultures that were negative for Shigella or EIEC were also negative by both of the hybridization studies [26].

Recommendations

Diagnosis of shigellosis is supported by the appropriate clinical presentation and the demonstration of sheets of white cells on fecal smears, but ultimately depends on identification of the organism in culture. The success of culture is enhanced by obtaining fresh specimens by proctoscopy early in the course of the illness, and by the use of proper transport media if plating is delayed. Serotyping is useful in epidemiologic studies but not in individual cases. Testing for the invasive virulence plasmid by DNA hybridization is in development. Use of biotinylated probes may simplify diagnosis, and may greatly shorten the time required, if sufficient sensitivity can be achieved to study stool directly without the need for prior isolation of

organisms. Low numbers of organisms in stool specimens, and their uneven distribution, may make clinical shigellosis less that ideal for the immediate application of these techniques.

Escherichia coli

At least four categories of *E. coli* strains cause diarrhea [27, 28]. These can be classified according to their district pathogenic mechanisms, and clinical presentations, into the enteropathogenic *E. coli* (EPEC), the enterotoxigenic *E. coli* (ETEC), the enteroinvasive *E. coli* (EIEC), the enterohemorrhagic *E. coli* (EHEC) and perhaps a fifth, recently recognized category, the enteroadherent *E. coli* (EAEC) [28]. Since all of these *E. coli* share growth and culture characteristics with the commensal *E. coli* strains which colonize the distal intestine, identification of pathogenic *E. coli* strains from fecal specimens represents a particular diagnostic challenge. Diagnosis depends on the recognition of particular serotypes associated with disease, or the identification of their individual virulence attributes. This is a difficult process, involving isolation and testing of a representative sample of 5–10 colonies from stool specimens. Diagnostic methods for pathogenic *E. coli* in stool are usually reserved for population and epidemiologic surveys, rather than for diagnosis of individual cases of diarrheal disease. *E. coli* serotype screening is performed less widely than in the past, and tests for *E. coli* virulence attributes are not widely available in routine laboratories. Molecular techniques have recently been applied to the diagnosis of *E. coli* infections through the application of DNA probes. These may be applied to stool samples without the need for colony isolation [1, 29–31].

Enteropathogenic E. coli

The enteropathogenic *E. coli* [32, 33] were the first *E. coli* to be associated with diarrheal disease when strains of particular serotypes were repeatedly associated with outbreaks of diarrhea in neonatal nurseries. EBEC diarrhea is now recognized more frequently in infants older than 30 days than in neonates, and presents as severe, chronic diarrheal disease with weight loss. Watery stools usually contain no fecal leukocytes.

The value of serotyping to diagnose EPEC infection has been repeatedly confirmed as strains of characteristic serotypes have continued to be associated with diarrheal disease over several decades. Serotyping includes determination of heat-stable O (somatic lipopolysaccharide) and heat-labile H (flagellar) antigen. Major O serogroups (class I) have been

defined as 055, 086, 0111, 0119, 0125, 0126, 0127, 0128ab and 0142 [28]. Less frequently associated minor serogoups (class II) include 018, 044, 0112 and 0114 [28]. Within these serogroups, disease is associated only with specific serotypes (e.g. 055:H6, 0111:H5). Initial screening of colonies obtained from stool, small bowel aspirate or biopsy is performed using commercial pools of antisera (Difco) for several serotypes. Colonies which screen positive should be confirmed by testing with individual specific O and H antisera. EPEC strains were initially reported to express unique heat labile capsular (K) antigens (designated B type), antibodies to which were absorbed out with heated cultures. It now appears that these strains do not produce true heat-labile acidic polysaccharide capsules; but instead produce a surface oligosaccharide related to the O (LPS) determinant [33]. Thus, older literature referred to B (or K) capsular determinants on these strains whereas current serotyping usually only designates O and H determinants. Recently, EPEC attempts have been made to associate the major and minor EPEC serogroups with particular virulence determinants [28].

EPEC Adherence Assays. EPEC strains are noninvasive but closely adhere to intestinal epithelium producing a characteristic lesion described as attaching, effacing enteroadherence [34]. These lesions of the individual epithelial cell can be identified by electron microscopy of small bowel or rectal biopsies. In vitro assays examining characteristic patterns of adherence of EPEC strains to cell lines in tissue culture (HEp2, HeLa) or to organ cultures of intestinal biopsies, have been developed [35, 36]. In particular, a pattern of localized adherence (LA) of EPEC microcolonies to HEp2 and HeLa cells is now recognized as a property of the major EPEC serotypes. Diffuse adherence (DA) over the entire surface of cultured cells is a less-specific property of minor serotypes. Characteristic attaching effacing EPEC lesions will develop within hours in intestinal organ cultures infected with EPEC. These assays have at times been used for epidemiologic characterization of *E. coli* isolates, but their primary utility is as research tools to localize and clone the genetic loci for EPEC virulence determinants.

Plasmids and Probes. The determinants for HEp2 localized adherence of EPEC are encoded by a 60- to 70-kdalton plasmid (termed pMAR2) in an 0127:H6 EPEC strain [37]. The pMAR2 plasmid (and homologous plasmids of similar size) are also necessary for full EPEC virulence in volunteer challenge studies. They encode for a presumptive adhesin termed EPEC adherence factor (EAF). A 23-kb Sal I fragment of pMAR2 has been cloned and used

as a probe for identification of EPEC [38]. This probe has been successful in identifying EPEC of the major serogroups listed above which show LA. Adherence genes in this fragment were cloned as two distinct plasmid regions of 1–5 kb which confer the adherence phenotype only when complementing each other [39]. Analysis of data from several labs, however, suggests that these plasmid encoded determinant(s) are neither necessary nor sufficient to produce the full characteristic attaching and effacing lesion in vitro. They may promote an early stage of attachment to the intact mucosal surface. The localization of the genes for EPEC effacing adherence and other virulence properties thus remains an area of intense research effort. EPEC strains do not produce LT or ST. Some strains previously considered to be EPEC, particularly those of serotype 026:H11, produce moderate to large amounts of shiga-like toxin (vero-toxin), but such strains may more properly belong to the EHEC category described below [28].

Enterotoxigenic E. coli

The enterotoxigenic *E. coli* are responsible for the majority of cases of traveller's diarrhea experienced within 1–2 weeks by 30–50% of visitors from industrialized countries to developing countries. These organisms are also a major cause of severe, and fatal, diarrheal illness in infants and children in developing nations. They induce a watery diarrhea without fecal leukocytes, which may be cholera-like in intensity. Transmission is by the fecal-oral route and contaminated food is the usual transmission vehicle. Although many animals (including pigs and cattle) experience enterotoxigenic *E. coli* infection, the strains infecting humans and animals are distinct in their expression of species-specific adherence determinants.

The ETEC strains fall into a limited number of serogroups which correlate with the expression of particular combinations of colonization factor antigens and toxins. Antisera against common ETEC O serogroups have been suggested as a means of simplifying ETEC identification in the clinical laboratory. In one study, two pools of antisera against 12 total serotypes had sensitivity of 64%, specificity of 96% and a predictive accuracy of 89% as compared to tissue culture enterotoxin assays [40]. Orskov and Orskov [41] have proposed adding the F (or fimbrial) antigen expression, as defined by crossed-line immunoelectorphoresis of saline extracts of fimbriated organisms, to the *E. coli* serotyping scheme. However, this has been difficult to establish in practice since expression of fimbrial antigens depends on growth conditions which are not standard for fimbrial types.

ETEC isolates from various sources produce both heat labile (LT) and heat stable (ST) toxins. These include LT-I, LT-II, ST-I and ST-II.

LT-I. The *E. coli* heat-labile toxin (LT-I) is closely related to cholera toxin with which it shares 75% nucleotide and 70–80% amino acid homology. Like cholera toxin, it consists of one 28-kdalton A (active) subunit and five 11.5-kdalton B (or binding) subunits. The A subunit activates adenylate cyclase through the ADP ribosylation of guanine nucleotide binding proteins. The B subunit binds to G_{M1} ganglioside on the surface of intestinal epithelial cells. Assays for LT-I include those which measure activation of adenylate cyclase in tissue culture cells; measure binding of LT-I to G_{M1} ganglioside; or depend on recognition of toxin by specific antibody. The genes for LT-I are located on plasmids, freqeuntly the same plasmids which encode for ETEC adherence factors. Recently, nucleotide probes have been developed for LT-I genes.

The Y1 mouse adrenal [42, 43] or Chinese hamster ovary (CHO) tests [44] utilize changes in tissue culture cell morphology (rounding) or steroidogenesis to detect *E. coli* LT activity in culture or stool filtrates. These tests were initally developed for cholera toxin activity but are equally applicable to LT. They provide the standard against which subsequent tests are measured for sensitivity and specificity. These tests have great advantages over rabbit ileal loops, but they require a tissue culture facility and are not useful for field studies.

The G_{M1}-ELISA, as reported by Svennerholm and Holmgren [45], is based on the specific binding of LT-I to polystyrene-absorbed G_{M1} ganglioside. Immunological detection of LT uses commercially available cross-reactive anti-cholera toxin antisera in an ELISA. This test and the G_{M1} HRP-ELISA [46] require analysis of supernates of individual colonies cultured in broth. In the field, the color development can be satisfactorily interpreted with the naked eye. An improved assay [47] optimizing culture conditions for toxin production (casamino acids) and toxin release from the periplasmic space (polymyxin B), achieved sensitivities similar to the Y1 assay. Direct culture of *E. coli* strains in the G_{M1}-coated wells of microtiter plates (so-called DCM-G_{M1} ELISA) can be performed.

In the Elek/Biken immunodiffusion test [48] a precipitin line forms in agar between a subcultured *E. coli* colony and anti-cholera toxin or anti-LT serum placed in an adjacent well. Colonies are grown for 48 h, polymyxin B added to lyse cells, and then the immunodiffusion reaction developed for 24–48 h. The test does not require isolation of culture filtrates and requires no special equipment for reading; however, it takes 5 days and precipitin lines may be subtle and hard to interpret.

Recently, Vadivelu et al. [49] described a membrane filter assay in which primary isolation of bacteria is performed on a cellulose-acetate membrane filter, after which the filter is transferred to an agar medium containing anti-cholera toxin. Zones of precipitation in the agar are read at 8 h. This assay permits examination of large numbers of strains without the need for subculture, pooling, preparation of supernates or radiolabelling.

A modified staphylococcal co-agglutination test [50] (Phadebact ETEC/LT) has recently been marketed by Pharmacia to detect LT extracted from isolated colonies of *E. coli*. This is based on formalin-treated and heat-killed *Staphylococcus aureus* which have been sensitized with high-titer rabbit anti-LT serum. In the two-step test toxin is extracted from colonies which may be picked from initial isolation plates, then the extract is agglutinated with the sensitized staphylococcal organisms.

LT-I Plasmids and Probes. The genes for LT-I A and B subunits have been cloned [51] on a 1.2-Mdalton fragment and subclones for A and B+A regions have been prepared for use as probes [31, 52]. These have been used to test isolated colonies as well as stool blots from patients with diarrhea. In general, the probes are highly sensitive for isolated colonies,

although they may give false positives (identifying strains which do not make enterotoxin) because of contamination with vector DNA. They are less sensitive for stool blots, and in particular for blots prepared in the field as opposed to in a central laboratory (presumably because of inadequate quality control in the fixation steps). A synthetic oligonucleotide probe of 20 bases [29] has also been prepared and evaluated for detecting LT genes in cultures and stool samples. The probe was sensitive for colonies but insensitive for testing stool samples directly.

LT-II. This heat-labile toxin also activates adenylate cyclase in YI adrenal cells via ADP-ribosylation and has A and B subunits similar in size to LT-I; however, this toxin is immunologically distinct from LT-I and is chromosomally, rather than plasmid, encoded [53]. The genes encoding this toxin have been cloned and a 0.8-kb DNA probe prepared which does not hybridize with structural genes for LT-I [54]. In a recent epidemiological survey of YI adrenal LT-positive *E. coli* strains from Southeast Asia using this probe, LT-II positive strains were commonly isolated from cattle, but rarely isolated from children with diarrhea [55].

Toxins designated ST-I (or ST_A) belong to a group, or family, of heat-stable, methanol-soluble peptides with molecular weights from 2–5 kdaltons that activate intestinal mucosal cell particulate guanylate cyclase. The active moiety is within a common C-terminal 18 amino acid segment [56] which contains six cysteine residues and seems to require cross-linking for activity.

The standard assay for the ST-I (ST_A) family is the in vivo suckling mouse in which culture supernates are injected transabdominally into the stomaches of suckling mice [57]. Intestines are later observed for fluid secretion which is quantitated as the ratio of the weight of the distended intestine to that of the carcass. One suckling mouse unit of toxin gives an intestine/carcass ratio of 0.08. The assay is cumbersome but reproducible. It is not sensitive for detecting ST directly in stools.

Giannella et al. [58] and Frantz and Robertson [59] developed radioimmunoassays for ST-I (ST_A) utilizing high-titer antisera raised by conjugating the poorly immunogenic toxin as a hapten to carrier protein, and using highly purified radiolabelled toxin. Because of the need for relatively large amounts of purified and highly labelled toxin, these assays have not been generally available. The assays recognized several types of ST-I (ST_A) but did not recognize ST-II (ST_B).

The degree of relatedness of the members of the ST-I family have been shown by gene cloning experiments of Moseley et al. [30, 60]. They first [60] prepared a radiolabelled fragment of DNA (estA1) encoding for an ST-I toxin designated ST-Ia from a pig isolate (hence also called ST-P). About half (15/43) of the suckling mouse positive strains isolated from humans hybridized with this probe under stringent conditions. Many more were positive under less-stringent conditions, suggesting related but not identical DNA sequences among the ST-I (suckling mouse positive) toxins produced by different *E. coli* strains. An additional probe (estA2) was prepared for an ST-I deignated ST-Ib from a human isolate (also called ST-H) which hybridized with the remainder of the test strains [34]. Some strains were detected by both probes. The genes estA1 and estA2 have 72% homology and their enterotoxins have 62% homology with highly conserved C-terminal regions. They do not hybridize with each other under stringent conditions. Thus, two probes are necessary to recognize the majority of ST-I producing isolates of human origin.

Since the above probes are restriction fragments of plasmid vectors containing cloned genes and they may theoretically give false-positive reactions due to contamination with vector

DNA. Murray et al. [29] evaluated small oligonucleotide probes of 22 bases synthesized according to the known sequences of ST-H and ST-P. They found good correlation between colony blots with the oligonucleotide probes and bioassy for ST-I; however, they found tests of stool growth instead of individual colonies difficult to interpret.

ST-I genes have been identified as being in a transposable element, accounting for their appearance on several different plasmids. In strains causing human diarrhea, STa and CFA/I production were encoded by the same plasmid, whereas a separate plasmid in the strains encoded LT-I.

ST-II (STb). This methanol-insoluble heat-stable toxin is negative in the suckling mouse assay but induces secretion in the pig or rabbit intestinal loop. This toxin is produced by strains pathogenic for pigs and cows, but has not been implicated in strains causing human disease. Less is known about its genetic organisation although it may also be on a transposable element.

Colonization Factor Antigens. The recognized colonization factor antigens (CFAs) and putative colonization factors (PCF) of the ETEC strains include both fimbrial (pilus) components (CFA/I, CS1 and CS2 of CFA/II, CS4 and CS5 of pcf8775) and finer fibrillar components (CS3 of CFA/II and CS6 of pcf8775). Expression of the several CFAs is associated with a limited number of serogroups (CFA/I 04, 01, 025, 063, 078, 090, 0110, 0126, 0128, 0153; CFA/II 06, 08, 09, 078, 080, 0115, 0139; pcf8775 025, 0115, 0167). The expression of these surface structures can be recognized because they confer the property of hemagglutination of human and bovin erythrocytes. This hemagglutination of ETEC CFAs is not inhibited by mannose, thus distinguishing these fimbrial components from type 1 of common pili. Both fimbriae and fibrillae are large macromolecular aggregates of smaller repeating protein subunits. Adhesive properties may reside in the major subunits, of in minor subunits. The expression of these adhesins depends on growth conditions and they are generally best expressed on the surface of agar. Orskov and Orskov [41] have proposed a serotyping scheme for fimbrial expression based on analysis of saline extracts of bacteria by crossed-line electrophoresis, but this has been difficult to implement because of the different growth conditions required for expression of different adhesins.

McConnell et al. [61] have developed an ELISA system for the expression of CFAs. They developed antisera specific for the several fimbrial and fibrillar components by immunizing rabbits with strains expressing individual adhesins, and absorbing with homologous adhesin-negative strains. Overall, the ELISA was more sensitive than hemagglutination of immunodiffusion. Identification of presently defined adhesins for ETEC will detect only about 40% of clincial isolates shown to be toxin producers by other tests.

The genes for several CFA components have been cloned including CFA/I [62] and CS3 [63]. However, these clones have not as yet been used to prepare nucleotide probes for clinical or epidemiological studies.

Enteroinvasive E. coli

The enteroinvasive *E. coli* are genetically similar to Shigellae and produce a similar clinical picture, dysentery or a watery diarrhea, but they differ from Shigellae in requiring a much higher inoculum to produce disease. They are nonmotile and slow or non-lactose fermenting. They are lysine decarboxylase negative and this has been suggested as a screening

test for selecting diarrheal isolates for further testing [64]. The characteristic 0 serogroups of EIEC are 028, 029, 0112, 0124, 0136, 0143, 0144, 0147, 0152, 0164 and 0167. These antisera are not readily available and they may identify nonpathogenic *E. coli* of the same serogroups. Thus, it is desirable to confirm the invasive phenotype by other tests.

The tests for EIEC invasiveness are those previously discussed for Shigellae. The Sereny test [20] (guinea pig keratoconjunctivitis) is definitive [64]. The indirect ELISA of Pal et al. [18] was developed using a high titer antiserum to a virulent EIEC (0143) absorbed with the Sereny-negative derivative. It can detect invasive clinical *E. coli* and shigella isolates, but it has not been useful to detect these organisms in stool samples directly.

EIEC and Shigellae possess large, 120- to 140-Mdalton plasmids which are associated with invasion [16]. Thus, one approach to identify EIEC is to screen isolates for large plasmids and then test them for invaseiveness by Sereny test or HeLa cell assay. This is an impractical way to handle large numbers of samples. A 17-kb EcoR1 fragment of the 140-Mdalton plasmid of *S. flexneri 5*, used as a probe, will differentiate EIEC from other *E. coli* in culture [23]. Recently, a biotinylated probe was developed which compares favorably with a previously tested radiolabelled probe [24].

Enterohemorrhagic E. coli

The EHEC strains of *E. coli* which are associated with outbreaks and sporadic cases of hemorrhagic colitis are among the *E. coli* which produce cytotoxins distinct from LT and ST. Community outbreaks have been associated with the ingestion of contaminated beef in fast food restaurants, and illness has also occurred in nursing homes and day care centers. Children and the elderly appear to be at greatest risk. These strains are also termed verocytotoxin (or shiga-like toxin) producing *E. coli* (VTEC). They were initially recognized as a novel serotype and then by the ability of their culture supernates to induce toxic effects on Vero cells or HeLa cells in tissue culture [65], effects which could be neutralized, or partially neutralized, by antibody to shiga toxin [66]. It is now known that these organisms produce two distinct shiga-like toxins (SLTs), called by some investigators verotoxins (VTs). SLT-I (VT1) is essentially identical to shiga toxin differing in only one amino acid in the A subunit. SLT-II (VT2) is only 60% homologous to shiga toxin based on nucleotide sequence. The genes for these toxins are carried on bateriophages [67], from which they have been cloned. Strains producing these toxins are also associated with nonbloody diarrhea, and with the development of hemolytic uremic

syndrome (HUS) felt to be related to effects of absorbed toxin on vascular endothelium [68]. Although fewer than 10% of patients with EHEC infection develop HUS, infection with 0157:H7 strains is the predominant cause of HUS in the US.

Serotyping. Enterohemorrhagic *E. coli* of serotype 0157:H7, the most common serotype, do not ferment sorbitol, thus screening for organisms of this serotype can be facilitated by selecting sorbitol-negative colonies for confirmatory serotyping and testing for SLT (VT) production after overnight incubation on sorbitol-MacConkey agar [69]. Additional, sorbitol-positive colonies should be selected for screening for SLT (VT) production by EHEC of other serotypes (predominantly 026:K60:H11 and 0103:H2, but also 0111:K58:H$^-$ and 0145:H$^-$) in a recent epidemiologic study of sporadic cases in Canada [70].

Toxin production is determined [65] by observing effects of culture supernates on Vero cells or HeLa cells in culture and neutralizing with appropriate antisera. Such tests are usually available in reference laboratories or those with special interest. ELISA and latex agglutination tests for demonstration of toxin production are being developed. The definition of globotriosylceramide (Gb_3) as the cell surface receptro for SLT-I should permit the development of receptor-binding assays analogous to the G_{M1}-binding assays for LT-I but these are currently less sensitive than cell culture assays.

These organisms can be demonstrated to be enteroadherent in an attaching and effacing manner as seen with EPEC strains. Their adherence factors may include a new fimbrial antigen encoded by a 60-Mdalton plasmid [71]. A 3.4-kb DNA probe has been prepared from this plasmid which hybridized with 99% of 0157:H7 strains and 77% of 026:H11 VT-positive strains [72].

Campylobacter

Once considered rare agents of infections in humans, Campylobacters are now among the common causes of bacterial diarrhea in man, and are isolated as often as Shigella and Salmonella in both developed and developing countries [73, 74]. Diarrhea occurs 1–7 days following oral ingestion of as few as 500 organisms and typically lasts less than 7 days, although persistent and relapsing symptoms can occur [75]. Diarrhea varies from watery stools to dysentery. Complications include Reiter's syndrome, reactive arthritis, and Guillain-Barré syndrome. Sources of infection include unpasteurized milk, improperly cooked poultry, and contaminated water. Campylobacters are commonly found as commensals in poultry, swine, sheep, cattle, dogs, and cats, and it is possible that the high optimum

growth temperature of *C. jejuni* reflects an adaptation to birds, which have high body temperatures. The taxonomy has changed recently: *Campylobacter fetus* spp. jejuni has been classified into two separate species, *C. jejuni* and *C. coli*. Although several species have been isolated from animals, *C. jejuni* and *C. coli* commonly cause diarrheal illness in humans. *C. fetus* spp. fetus is a relatively rare cause of human disease (often manifest only as septicemia) which chiefly attacks debilitated patients. Two other groups of spiral organisms are implicated in human disease and are included in the Campylobacter genus but will not be discussed here: *C. pylori* has been found colonizing the gastric mucosa of patients with gastritis. They may be more closely related to Wolinella than to other campylobacters [76]. The second group of Campylobacter-like organisms (CLOS) include *C. cinaedi* and *C. fennelliae* [77] and have been isolated from rectal swabs or biopsy specimens from symptomatic and asymptomatic homosexual men.

Procedures

Campylobacters are small (1.5–3.5 μm in length, 0.2–0.4 μm in width), nonspore-forming gram-negative organisms that have a characteristic curved, S-shaped, or spiral morphology (hence their name – "curved rod" in Greek) that permits their distinction from other gram-negative roads on microscopic examination of fecal smears. The cells possess a single flagellum at one or both poles, and they move with a characteristic rapid corkscrew-like darting motion when observed with phase-contrast microscopy.

Transport media, including Campy-thio, Carey-Blair (thioglycolate), Ames or Stuart's modified thioglycolate, are useful and permit survival for 1 week or longer especially if refrigerated [78]. Buffered glycerol saline is unsuitable [79]. Campy-Thio can be inoculated with 3–5 drops of liquid stool, or a rectal swab can be inserted about 1 cm into the medium and twirled to allow inoculation of the organisms into a zone of reduced oxygen. After overnight refrigeration in Campy-Thio, the inoculum for subculture should be withdrawn by inserting a Pasteur pipette 2 cm below the surface and continually aspirating during withdrawal. A few drops of this aspirate are then placed on a selective media and streaked for isolation.

C. jejuni in pure cultures grow best at 42 °C in microaerobic environments (5–10% oxygen and 3–10% carbon dioxide) on a variety of basal enteric media including Mueller-Hinton and brucella agars. However, isolation from stool specimens requires direct inoculation of the specimens onto a selective antibiotic media supplemented with various nutrients (such

as Campy-BAP), or indirect inoculation into an enrichment broth (such as Campy-Thio) (thioglycollate broth with 0,16% agar and the antimicrobials used in Campy-BAP) for overnight refrigeration with subsequent subculture to a selective medium, since campylobacters are rapidly overgrown by the other organisms in fecal specimens.

Incubation cultures at 42 °C allows for more rapid growth of *C. jejuni/C. coli,* and appears to inhibit growth of some fecal flora, but has the disadvantage of inhibiting growth of *C. fetus.* However, *C. fetus* is rarely isolated in stools, and is basically an opportunistic organism associated with disease in immune-compromised hosts [79].

Several different selective media that contain three or more antibiotics to retard the growth of other organisms are in common use. The three most commonly used media are campy-BAP, Skirrow's, and Butzler's medium Oxoid [79, 80], and are available from BBL, Remel, Gibco, and Scott. Two other described media are: Preston medium, reported to be as sensitive but more selective and less expesive than Skirrow's, and Butzler's Medium Virion which is reported to be comparable to Butzler's Medium Oxoid in sensitivity, but more effective at suppressing competing enteric flora and permiting isolation at 37 °C. Two distinct colony types have been described: a flat, grayish, spreading nonhemolytic colony with an irregular shape, and round, convex colonies with an entire edge. The moisture content of the medium may determine which colonial morphology occurs [81].

An early selective method for isolating Campylobacter that is still useful, particularly in countries where the antibiotics for selective media are not readily available, is based on filtration and depended on the small size of campylobacters for separation from other bacteria. Steele and McDermott [82] modified Dekeyser's filtration method [83] by directly applying a 0,45-µm (Millipore) filter onto nonselective media. 8–10 drops of a heavy suspension of stool in saline is applied to the filter for 30 min at room temperature. The filter is removed and the media incubated for 24–48 h at 42 °C under microaerobic conditions. Other enteric organisms are removed with the filter, but because of their small size, campylobacters pass through. Theoretical advantages of this method are that inhibition of Campylobacter growth by antibiotics does not occur, and the large inoculum may permit an enrichment effect.

The classical approach of serotyping gram-negative organisms based on heat-stable O antigens and heat-labile H and K antigens has been less successful for Campylobacter than for other enterics because of autoagglutination and non-specificity after heating. Complex serotyping schemes

based on either heat-labile or heat-stable antigens are not fully standardized or available commercially. The Lauwers and Penner [84] systems which are both passive hemagglutination assays, attempt to avoid the problems of nonspecificity by extracting heat-stable (presumably O) antigens and sensitizing red blood cells with them. At least 50 different serotypes have been identified in these systems. The Lior [85] system, which is a slide agglutination assay based on heat-labile antigens (probably flagellar or capsular antigens), reduce the number of nonspecific reactions by absorbing antisera with homologous heat-stable antigens. Thirty-six serogroups have been identified by this system.

Several tests have been developed to detect the development of serum antibody to Campylobacter, including a serum bactericidal assay, agglutination tests, indirect fluorescent antibody test, enzyme immunoassays, and a complement fixation test [86–88]. A limitation of most of these tests is that they react with strain-specific antigens, and therefore require that a bacterial isolate from at least 1 patient involved in an outbreak be used as an antigen. The antigen for the complement fixation test, however, is prepared from a random mixture of serotypes and reacts with group-specific antigens, obviating the need for a homologous or related strain (Virion CF Test, Morristown, N.J.).

Plasmids have been detected in less than 20% of Campylobacters [89], diminishing their potential importance for rapid identification or epidemiology. Tompkins et al. [90] have reported on the use of a variety of chromosomal DNA probes, including fragments of *C. jejuni* chromosomal DNA, specific endonuclease restriction fragments of *C. jejuni* chromosomal DNA cloned in pBR322, and single stranded synthetic oligonucleotide probes. A *C. jejuni* chromosomal fragment radiolabeled by nick translation was shown to hybridize with *C. jejuni*, but not other Campylobacter species or other enteric organisms. This probe was used to detect campylobacters in 299 diarrheal stools dotted directly to nitrocellulose filters (without growing the colonies) and successfully identified 10 of 18 stools from which *C. jejuni* was isolated by culture. Although specific, sensitivity was low, requiring at least 1,007 organisms per milligram of stool.

Recommendations

Fresh fecal specimens should be directly plated onto selective media or inoculated into broths such as Campy-thio for transport and enrichment. A fecal smear showing typical morphology is helpful. A presumptive identification may be based on growth on selective media incubated at 42 °C under microaerobic conditions, typical colonial and gram stain morphology, motility, and positive oxidase and catalase tests. The filtration technique may permit isolation if selective media are not available. The DNA

hybridization assay is insensitive, not commercially available, and presents the inherent problems involved in handling and maintaining radioactive reagents.

Yersinia

The importance of *Y. enterocolitica* as an enteric pathogen was emphasized in 1976, when an epidemic of gastroenteritis involved 222 subjects and resulted in over a dozen children undergoing appendectomies [91]. Yersinia is now consistently isolated in 8–10% of diarrheal stool specimens in some northern geographic areas [92]. Several clinical forms of Yersinia enteritis have been recognized, including acute and chronic (lasting longer than 1 week) diarrhea, acute abdominal pain resembling appendicitis, localized infections, and septicemia [93]. The majority of patients with proven disease have been children. Children younger than 6 years old tend to have diarrheal illness while older children may have abdominal pain as the dominant feature. The diarrheal illnes may begin with 1 or 2 days of watery stools that progresses to blood-streaked stools with tenesmus, right lower quandrant tenderness, fever, myalgias, and headache. About 50% of children and 10% of adults also experience pharyngitis. Postinfectious complications that have been reported include thyroiditis, glomerulopathy, polyarthritis, Reiter's syndrome, erythema nodosum, erythema multiforme, and carditis. Children with chronic diarrhea due to Yersinia are often suspected of having idiopathic inflammatory bowel disease due to similarities in presentation. There are three Yersinia species that are pathologic for man. *Y. enterocolitica* and *Y. pseudotuberculosis* are primarily intestinal pathogens, and *Y. pestis,* the agent of bubonic plague, has a greater tendency to cause fulminant bacteremia and suppurative lesions of the lymphatic system. Two accepted methods for typing strains of *Y. enterocolitica* are serotyping (based on bacterial agglutination with rabbit anto-0 antisera), and biotyping (defined by Wauters and based upon the use of indole, xylose, and trehalose and the presence of lipase and DNAase) [93]. The predominance of human serotypes varies with location. Serotypes 0:5, 0:8, and 0:9 are the usual isolates in the United States, but 0:3, 0:5, and 0:8 are most common in Canada and 0:3 and 0:9 in Sweden. Of the five biotypes, most human strains are biotype four [93]. *Y. enterocolitica* and *Y. pseudotuberculosis* have a worldwide distribution, but *Y. pseudotuberculosis* is rarely isolated in the United States.

Procedures

Standard laboratory diagnosis involves culture of the organism, followed by biochemical and serologic tests if necessary. Presumptive identification by culture on usual enteric media such as Hektoen enteric, MacConkey, eosin-methylene blue, and blood agar depend on an awareness of the pinpoint morphology of the colonies and temperature-dependent growth characteristics. They produce an acid/acid reaction on triple sugar iron slants, ferment glucose but not lactose, and procedure ornithine decarboxylase. Human strains are usually sucrose positive, rhamnose negative, and melibiose negative. The colonies are nonpigmented and organisms are motile at 22 °C. Several selective and differential agar media have been developed to enhance recovery of *Y. enterocolitica* from stool specimens [94]. On Yersinia selective (Schiemann CIN) media, colonies have a "bull's eye" appearance with a deep cherry-red center and transparent margins and have a distinctive odor. Cold enrichment (4 °C) of stool specimens in phosphate-buffered saline with weekly subculture enhances recovery from stool specimens, and this is useful when organisms are shed in small numbers such as during the covalescence or in detecting carriers [93].

Serology has been useful as a diagnostic adjunct only in well circumscribed outbreaks of Yersinia gastroenteritis [95]. The use of serology in individual cases is limited by a paucity of data about antibody prevalence, as well as by the unavailability of Yersinia antigens for performing these assays.

A rapid method to distinguish pathogenic *Y. enterocolitica* isolates from nonpathogenic Yersinia strains would be useful in epidemiological surveys, and one proposed virulence marker is a plasmid-independent 24,000-dalton surface protein [96]. A coagglutination assay to detect this protein using staphylococci coated with specific antiserum has recently been reported to correlate 100% with tested pathogenic biogroups [96].

The molecular genetics of Yersinia species have not been widely exploited for diagnostic purposes, although restriction maps have been made for the virulence plasmids from several *Y. enterocolitica* serotypes (0:3, 0·8, and 0.9), and the plasmids are reported to be closely related, sharing functional similarities and high degrees of DNA homology, making the development of DNA probes conceivable [97].

Recommendations

Diagnosis depends on isolation of the organisms in culture, and is enhanced by the use of Yersinia-selective media. If pharyngitis is present,

throat cultures should be obtained. Patients with diarrhea usually excrete sufficient numbers of organisms so that cold enrichment is of marginal benefit except to identify carriers. Recognition of typical colonial morphology on enteric media is facilitated by the use of a stereomicroscope. Serotyping and biotyping can be performed by reference laboratories if necessary. Identification of virulence factors such as the 24,000-dalton surface protein by coagglutination of antibody-coated staphylococci, or DNA probe hybridization are as yet restricted to the research laboratory.

Clostridium difficile

C. difficile was not recognized as an enteric pathogen prior to the late 1970s. *C. difficile* is now recognized as the most important cause of antibiotic-associated colitis, and is one of the most commonly diagnosed enteric pathogens in hospital practice [98]. The spectrum of presentation ranges from a mild, watery diarrhea to hematochezia with high fever, severe abdominal pain, localized tenderness, and polymorphonuclear leukocytosis. Illness from *C. difficile* infection rarely occurs without a prior history of antibiotic administration (especially clindamycin, ampicillin or cephalosporins, but rarely vancomycin or parenteral aminoglycosides) and the incidence of infection has been shown to correlate with the presence of spores in the environment [99]. Recent advances in laboratory diagnosis have been limited to the development of tests for toxins in stool samples.

Procedures

Clinically, the diagnosis of *C. difficile* infection can be made with remarkable certainty if typical yellow-white pseudomembranous plaques are seen overlying the mucosa at proctosocopy, flexible sigmoidoscopy, or colonoscopy [98], since almost all (99%) cases of pseudomembranous colitis thus recognized are caused by *C. difficile*. A failure to demonstrate pseudomembranes on sigmoidoscopy does not rule out the diagnosis, since the distribution of these lesions may be patchy with rectal sparing [100].

Gram-stained fecal smears are not helpful for diagnosis because of the uneven distribution of organisms in specimens and difficulties in judging a predominance of gram-positive rods [101]. Wilson et al. [102] detected *C. difficile* in smears by direct immunofluoresence in 26 (81%) of 32 stool specimens that were also positive by culture or toxin B assay, but were unable to distinguish between pathogens and cross-reacting clostridial species.

Laboratory diagnosis depends on successful culture of *C. difficile* and or the detection of *C. difficile* toxins in stool filtrates. Isolation of *C. difficile* in pure culture was formerly difficult (hence its name), since it has few colonial morphological characteristics that distinguish it from other more numerous anerobes on artificial media. The development of a commercially available selective anaerobic medium (CCFA) by George et al. [103] containing cycloserine, and cefoxitin has greatly improved the efficiency of detecting the organisms from patients who have more than 1,004 organisms per gram of stool. Rapid plating of specimens is required, since storage, freezing, or exposure of samples to air before plating diminishes recovery. On selective media the organisms grow in yellow, circular colonies with a filamentous edge, which emit a golden yellow fluorescence under ultraviolet light. The addition of sodium taurocholate to selective agar improves the detection of the spore forms, which is important in monitoring soil or hospital environments [104, 105].

A number of difficulties hinder the interpretation of stool cultures, which are usually not quantitative and are reported as positive or negative. The most important problem is that *C. difficile* can be detected in stools from healthy, asymptomatic carriers [106]. 1–5% of adults are carriers, and even higher isolation rates (10–60%) have been found in apparently healthy infants in newborn nurseries [106]. These findings are partially explained by the fact that not all *C. difficile* isolates are toxin producers and therefore pathogenic [107]. This is not the whole explanation, however, since high toxin producers have been isolated from infants who are apparently well, suggesting that some infants may be resistant to the effects of toxin. Because of these difficulties, many laboratories will only perform cultures on diarrheal specimens if a clinical history of antibiotic usage is provided. Finally, a 48-hour delay is required for even a presumptive diagnosis by CCFA culture.

C. difficile produces at least two toxins: A, called an enterotoxin (because of its affect on animal loops), and B, called a cytotoxin (because of its effect on tissue cultures). The importance and exact role that each plays in the etiology of *C. difficile* diarrhea has not been fully defined, in part due to difficulties in separating the toxins in pure form. The detection of toxin B in stools continues to be a standard laboratory test for *C. difficile*. Although most adults with *C. difficile* cytotoxin B in their stools have colitis and fewer than 2% with pseudomembranous colitis are negative [105] for toxin B, its importance has come under question. Although toxin B induces rounding of tissue culture fibroblasts, probably by depolymerization of actin-containing thin microfilaments, it has no effect in intestinal loops. Toxin A, on the other hand, does cause secretion of fluid in ligated rabbit ilea loops, where it elicits severe intestinal inflammation and alteration of epithelial permeability [108]. Toxin A

may prove to be more important than toxin B [108], but assays for its detection are not easily performed.

Detection of *toxin B* is easier than anaerobic culture, and, when properly performed, is quite sensitive and specific [98]. The stool specimen is homogenized, centrifuged, and sterilized by filtration. The filtrate is then added to a monolayer of tissue culture cells (usually fibroblasts) and the cells observed with an inverted phase microscope at 24 and 48 h. Picogram amounts of cytotoxin induce rounding of the cell bodies and thinning of the cytoplasmic processes that extend from the cell and attach it to the surface of the culture dish. Specificity is demonstrated by neutralization of cytoxicity by pre-incubation the filtrate with *C. difficile* antitoxin. A commercial kit for detecting cytotoxin based on the above method ("Toxi-Titer" Bartels Immunodiagnostic Supplies, Inc., Bellevue, Wash.) has become available and allows laboratories not routinely performing tissue culture assays to test for *C. difficile* toxin B [109]. The kit includes a toxin control, antisera against *C. difficile*, a diluent for preparing the stool filtrate, and a 48-well microtiter plate seeded with a 75–100% confluent growth of viable human foreskin fibroblast cells, ready for the cytotoxicity assay without additional processing. This kit was recently compared to conventional tissue culture assays by two laboratories and generally found to be satisfactory [109, 110].

Detection of *toxin A* has lagged behind assays for toxin B due to difficulties in purification and isolation. A latex agglutination kit was recently marketed for rapid detection of toxin A (Culturette Brand Rapid Latex Test Mitsubishi Chemical Industries, Tokyo, Japan; distributed by Marion laboratories, Kansas City) [111]. However, further efforts with purification and separation procedures indicated that the test reacted with an unidentified antigen and is not a test for enterotoxin A production, at least for some strains of *C. difficile* [112]. Paradoxically, the test has some clinical utility in that it was comparable in sensitivity to the cytotoxin assay in patients meeting clinical criteria for *C. difficile*-related antibiotic-associated diarrhea and colitis [113].

Several other laboratory tests for *C. difficile* are reported but of undetermined clinical usefullness. Initial reports of counterimmunoelectrophoresis (CIE) using *C. difficile* culture filtrates and detecting antigens with *C. sordellii* antitoxin suggested that this relatively rapid, simple, and inexpensive test was more sensitive with fecal specimens than were cytotoxin assays [114, 115]. However, impurity of antisera and cross-reactivity with nonpathogenic Clostridial antigens resulted in too frequent false-negative and false-positive tests for the test to be useful in screening or diagnosis [116–118]. Gas-liquid chromatography has been used to detect a fatty acid metabolite of *C. difficile* in stools from patients and controls, and although specificity was good, sensitivity was only 61% [119]. Serotyping has not been useful. A bacteriophage and bacteriocin typing scheme is under development for use in epidemiological investigations, but is unlikely to be widely used for routine diagnostic purposes [120]. Another potentially useful tool for epidemiological studies involves a typing method based on polyacrylamide gel electrophoresis of ^{35}S-methionine-labeled proteins [121].

Recommendations

Patients with onset of diarrhea after starting a course of antibiotics (especially clindamycin, ampicillin, and cephalosporins) should have flexible sigmoidoscopy if practical and specific therapy initiated if typical pseudomembranes are seen. Full colonoscopy increases detection of

pseudomembranes from 30% of antibiotic-associated colitis patients to 85% [122] but is not recommended in severely ill patients. Microscopic examination of stools is not helpful. Specimens for culture should be obtained during regular lab hours to permit rapid plating for enhanced recovery. Satisfactory tests for cytotoxin B are commercially available in kit form. While the "Culturette Brand CDT" *C. difficile* test apparently does not detect enterotoxin A, it does appear to have clinical usefulness and permits a presumptive diagnosis in hours instead of days.

Vibrio parahemolyticus

V. parahemolyticus are pleomorphic, halophilic gram negative rods of the family Vibrionaceae. These organisms are present in the marine environment and are responsible for outbreaks of gastroenteritis following the ingestion of raw or inadequately cooked fish or shellfish. Such outbreaks have been well recognized in Japan. In the US they have been associated with the ingestion of crabs or shrimp. The incubation period of the illness is 9–24 hours. The organism produces small superficial colonic ulcerations and the presence of fecal leukocytes can be demonstrated. Illness can present as cholera-like watery diarrhea or as a dysentery syndrome.

Procedures

V. parahemolyticus is isolated on TCBS (thiosulfate citrate bile salts sucrose) where the colonies have a blue center. Enrichment can be accomplished in Cary-Blair or alkaline peptone water with increased salt concentration. The organisms differ from other vibrio species in their lateral flagella, growth at 40 °C, utilization of putrescine and lack of swarming on complex media. A serotyping scheme based on O and K antigens has been developed. Pathogenic organisms (96% of clinical isolates) have been associated with the production of a thermostable hemolysin which induces the Kanagawa phenomenon (KP) or hemolysis of type O RBCs on Wagatsuma agar. 99% of environmental isolates lack this hemolysin.

A gene probe for this hemolysin, which is chromosomally determined, has been prepared and tested in a dot colony hybridization test (DVH) [123]. DCH is more sensitive than the Kanagawa test for detecting the hemolysin and compares favorably with immunological tests including the ELISA and modified ELEK (immunoprecipitation) tests [124].

Recently, strains hemolysin negative by gene probe and immunological testing (all serovariant O3:K6) were isolated from an outbreak of Traveller's diarrhea in the Maldives [125], thus other pathogenic properties may actually be important for virulence. However, this study must be interpreted in light of the demonstrated instability of the hemolysin gene in culture

Recommendations

This organism should be sought when there is a recent history of seafood ingestion, particularly during the summer months. The presentation may include evidence of active colonic inflammation. Tests for the thermostable hemolysin, including the gene probe, are not routinely available in the US.

Aeromonas Species

The species of the genus Aeromonas are gram-negative, rod-shaped, oxidase-positive, facultative anaerobes with polyflagellae currently listed in the family Vibrionaceae [126]. These organisms are present in fresh water and sewage and include a nonmotile group (salmonicida) which are fish pathogens. Species implicated in human disease are motile with single polar flagellae.

Isolation of Aeromonas from stool as predominant organisms has been associated with sporadic cases of diarrhea, but not with outbreaks. They have been associated with Traveller's diarrhea in Thailand [127], with summer diarrhea in Australian children [128] and in neonatal diarrhea. Taxonomy has been evolving. The current edition of Bergey's manual [126] divides the genus Aeromonas into the three species: hydrophila, caviae and sobria based on biotyping and analysis of DNA relatedness groups [129]. The species hydrophila and caviae produce potential virulence factors [130, 131] but the relation of these factors to enteric pathogenesis remains unresolved.

Procedures

Aeromonads survive in hypotonic media, in buffered glycerol saline or Cary-Blair transport media. They grow well on primary non-selective media, such as sheep blood agar. Optimum growth is at 28 °C. Since most strains are ampicillin resistant, ampicillin addition to blood agar plates provides a selective medium. Enrichment can be achieved in peptone water, or by maintenance at 4 °C for 24 h in buffered saline. These

techniques are of primary importance for isolation from environmental sources, convalescent patients or possible carriers.

The organisms appear similar to *E. coli* on blood agar and on Hektoen enteric medium. Since they are oxidase positive, representative colonies on nonselective media should be screened for oxidase activity by flooding areas of the primary isolation plate with oxidase reagent turning Aeromonas, Plesiomonas and vibrios dark purple. This is the fastest test to differentiate from *E. coli,* and it is also more practical than the use of ampicillin selection, since it gives an estimate of the predominance of colonies. Some labs only report Aeromonas if the organisms are heavy on nonselective media. Sensitivity testing on isolates representing fewer than 5% of colonies will usually require a specific request from the clinician.

Oxidase-positive colonies can be confirmed by API 20E testing and by sensitivity to compound 0/129 (2,4-diamino-6,7-diisopropylpteridine). Kaper et al. [132] described a multitest screening medium which combines mannitol (positive) and inositol (negative) fermentation, ornithine decarboxylation (negative) and deamination (negative), indole production (positive), motility (positive) and H_2S production. Species determination of confirmed isolates can then be made by use of: Voges-Proskauer reaction (variable with sobria, positive for hydrophila); H_2S production from cysteine (positive for hydrophilia and sobria); gas from glucose (positive for hydrophilia and sobria), esculin hydrolysis (positive for caviae and hydrophila) and salicin fermentation (positive with caviae and hydrophila). The three species have DNA homology values of 35–50%. Popoff et al. [129] have described 3 DNA hybridization groups in *A. hydrophila;* 2 in *A. caviae* and 2 in *A. sobria;* representing 70–90% relatedness among the groups in each species. At present, these groups within a single species cannot be differentiated by phenotypic characteristics.

Aeromonas species produce a variety of potential virulence factors [133] including: (a) hemolysins: demonstrable in culture supernates with 1% suspensions of rabbit erythrocytes in microtiter wells, or by a plate method with 5% sheep blood in tryptic soy agar; (b) cytotoxin, demonstrable in cell free supernatants against Vero cells [134], (c) heat-labile (56 °C, 10 min) enterotoxin; active against Y1 adrenal cells or demonstrable in the suckling mouse assay comparing intestinal weight to remaining body weight after intragastric injection (positive greater than 0.08) [128]; or in a 16-hour rabbit ileal loop assay [135].

In a recent study [130], none of these factors were found with caviae strains, all were found with hydrophila strains, whereas sobria produced only hemolysin and cytotoxin. This is consistent with the association of hydrophila and caviae isolates with diarrhea.

Discussion

Whether Aeromonas species represent true enteric pathogens has been controversial. Epidemiologic studies of the association of Aeromonas species with diarrhea must be interpreted in light of whether they were isolated from stool with enrichment techniques and selective media, or

whether they were predominant organisms on direct plating. Small numbers of organisms in the stool may simply reflect the characteristics of the local water supply. In some areas of the United States, e.g. Michigan, Aeromonas are not found in normal stools even with selective media [136]. When rigorous isolation techniques were used in Thailand, Aeromonas was isolated frequently (25–30%), and equally often from Thais with and without diarrhea [127]; however, at the same time, they were isolated more often from Peace Corps volunteers with diarrhea than from those without diarrhea.

Because of the uncertain clinical importance of isolating of small numbers of Aeromonas from the stool, we recommend that primary growth be performed on nonselective media, such as sheep blood agar, without enrichment. This is in accord with the findings of Robinson et al. [137] that 89% of enterotoxigenic strains isolated from direct plating were associated with diarrhea; whereas only 21% of those isolated after enrichment were associated with diarrhea, even without ampicillin. In an Italian study of childhood gastroenteritis [138] ampicillin plates yielded no significant difference in isolation rates between diarrheal (3.7%) and nondiarrheal stools (2.1%). We agree with Holberg at al. [139] that the "determination of the incidence of these bacteria as a possible cause of enteric disease will depend on the ability and willingness . . . to test predominant colonies on nonselective media for oxidase positivity".

Not all authors agree with the recommendation to use nonselective media. Agger et al. [136] studied nonselective sheep blood agar vs. the selective medium sheep blood ampicillin (SBA) and concluded that SBA gave increased frequency of isolation but only ir specimens from patients with diarrhea. Similarly, Kay et al. [140], working in Peru, recommended selective media and enrichment in peptone water for initial isolations. Such recommendations may depend on the low prevalence of Aeromonas in local water sources.

Interpretation of epidemiologic studies are complicated by the fact that, although a number of potential virulence factors have been described in Aeromonas; their production has not been conclusively related to disease. In Australia during the summer, Gracey et al. [128] reported that enterotoxin-producing Aeromonas (as defined with a suckling mouse assay) were found much more often in stools from children with diarrhea (10.2%), than in those from well children (0.6%). At the same time, the isolation rate of all Aeromonas, using blood agar containing *p*-nitro phenyl glycerine (and later plus 10 µg ampicillin/ml), was similar in the well and ill

group children. In contrast to this evidence suggesting a pathogenic role for enterotoxin, volunteer challenge studies with strains known to produce various putative virulence factors, have failed to produce disease in healthy North American volunteers [141]. All strains tested in volunteers were positive in rabbit ileal loops; all produced cytotoxin, hemolysin, enterotoxin and DNAase; but all were Sereny test and hemagglutinin negative. Until our understanding of Aeromonas virulence factors is improved, epidemiologic studies of Aeromonas isolation rates are unlikely to determine whether Aeromonas is an enteric pathogen. Moreover, tests for virulence factors cannot currently be recommended to confirm pathogenicity of individual isolates.

Viruses

In the 1970s, identification of the medically important viral etiologies of enteric disease relied on direct detection of virus particles in stool specimens by electron microscopy or immunoassays. Since that time, major advances have occurred in the development of highly sensitive, specific, and rapid solid-phase immunoassay techniques, several of which are commercially available. It is now possible to identify a causative virus from a diarrheal stool within a few hours, a much shorter time than is required to identify Salmonella, Shigella, or other common bacterial pathogens. This has practical significance, particularly for pediatric patients, in that a positive diagnosis eliminates the need for costly broad-spectrum IV antibiotics, permits reassurance in terms of prognosis, and facilitates appropriate disease spread-control measures.

Acute viral gastroenteritis is an extremely common illness (second only to the common cold in the US) that affects all age groups and occurs in epidemic and endemic forms [142–144]. The clinical syndrome varies, but is generally rapid in onset, self-limited, and characterized by various combinations of diarrhea, nausea, vomiting, and systemic symptoms such as low-grade fever, headache, abdominal cramps, myalgias, and malaise. Bloody diarrhea is not a feature of viral enteritis. Thus, if a fecal smear reveals sheets of inflammatory cells, a viral etiology is probably excluded. Three major groups of viruses, rotaviruses, Norwalk-like viruses, and enteric adenoviruses, are now unequivocally recognized as medically important. The reported frequency of occurence of the groups is influenced by age of the patients studied, the geographical location, and the season. Other

diarrhea-associated viruses, such as calicivirus, astrovirus, and coronavirus, can be identified, but their medical importance is uncertain.

Rotaviruses

Rotaviruses (family Reoviridae) primarily affect children, causing approximately half the episodes of infantile diarrhea that require hospitalization, but also cause illness in adults [145]. A new classification scheme groups Rotavirus into five groups (A–E) based on antigenic properties, genome profiles, and nucleotide sequences [146]. Thus far, human strains have all been in groups, A, B, or C, with the usual agents of childhood gastroenteritis being in group A [146]. Diagnostic tests include electron microscopy, solid-phase immune electron microscopy (SPIEM), counter-immunoelectrophoresis, polyacrylamide gel electrophoresis of viral RNA, radioimmunoassay (RIA), ELISAs, latex agglutination [142], and dot hybridization [147].

Procedures

Rotaviruses were orginally discovered in biopsies of proximal small intestine from hospitalized infants, and detection of virus in biopsy or stool specimens by electron microscopy remains the standard by which new tests are compared. Rotaviruses are 70 nm in size, contain dsRNA, and survive low pH (pH 3), heating (50 °C), and most detergents and solvents. Electron microscopy requires expensive equipment and highly trained technicians, and is impractical for screening large numbers of samples, but can detect atypical viruses using convalescent sera. Serologic assays are helpful in epidemiological studies, but are not helpful clinically since detectable pre-existing rotavirus antibodies are found in most individuals. Furthermore, the need for seroconversion prevents diagnosis early in the illness. Although 50% of field strains which have been detected can now successfully be grown in cell culture, this is time consuming and cumbersome. Therefore, the several variations of solid-phase immunoassays are the diagnostic tests of choice for most situations [148–151].

A partial list of the commercially available assays includes: Rotazyme (Abbot laboratories, North Chicago, Ill.), Enzygnost (Behring Institut, Marburg, FRG), Rotolex (Orion Diagnostics, Helsinki, Finland) and Rotoclone which uses a monoclonal antibody. The most critical factors for the performance of solid-phase immunoassays are the antibodies, but

convalescent human sera are not of sufficiently high titer for practical use. This difficulty has been overcome by growing group A animal strains (which cross-react with human strains) to high titers in cell culture for the preparation of hyperimmune diagnostic sera. Thus, commercially available assays detect group A antigens. Few non-group A rotaviruses have been detected in humans, but it should be noted that two outbreaks of group B rotaviral diarrheal illness (as determined by ELISA and genomic analysis) affected adults as well as children in China, indicating a need to determine the global significance of the B viral group in humans [145, 152].

A nucleic acid dot hybridization technique applied to the detection of rotavirus in stool specimens is reported to be specific and 10–100 times more sensitive than a conventional ELISA [147].

Recommendations

All assays are sensitive and specific, and the choice depends somewhat on the needs of the laboratory. For example, the Rotazyme test is performed with polystyrene beads as the solid phase in plastic test tubes and is read in a standard spectrophotometer, making it a convenient assay to use when small numbers of samples are processed. Enzygnost is performed in microtiter plates, permitting the use of automated equipment for the handling of large numbers of specimens [153]. A DNA hybridization test is not commercially available and not likely to replace the more convenient immunoassays currently in clinical practice, since it has the disadvantages of using short-lived radioisotopes that require special handling.

Norwalk-Like Viruses

The Norwalk-like viruses are a frequent cause of diarrheal illness (particularly in older children and adults), and usually occurs in epidemics. The Norwalk virus was the first virus to be convincingly associated with human gastroenteritis and was discovered by Kapikian et al. [154] in 1972 by immune electron microscopy (IEM) studies on stool specimens obtained during a diarrheal epidemic in Norwalk, Ohio. These viruses are shed in small amounts and for only a short time after the onset of diarrheal illness (3 days). Other viruses included in this group are the Hawaii, Marin County, Ditchling, "W", Snow Mountain, Parramatta, and cockle agent [155]. At least four distinct serotypes have been identified by immune electron microscopic studies of antigen relatedness.

Procedures

Norwalk-like viruses can only be visualized by the laborious and technically difficult procedure of immunoelectron microscopy, and the development of other diagnostic tests has lagged far behind developments for Rotavirus. These viruses are smaller (27 nm) than rotaviruses, round in shape, and the composition of their nucleic acids is uncertain, although studies of the proteins of purified Norwalk virions indicate that this virus is related to RNA-containing caliciviruses [155]. The virus has not been cultivated in vitro or in laboratory animals, and unlike Rotavirus, has no known cross-reactive substitute antigens that can be produced in highly purified forms, preventing production of hyperimmune serum reagents in animals. Therefore, the critical reagents for Norwalk virus immunoassays must be derived from serum or stool samples from human volunteers who have been infected with Norwalk virus, and this significantly limits their availability.

Recommendations

A radioimmunoassay is in use for detection of Norwalk virus in stools and for the quantitation of antibodies to the virus in sera and intestinal fluids, but is limited to the few research laboratories that possess limited amounts of the valuable human diagnostic reagents [156, 157]. The recent development of a monoclonal antibody to the Snow Mountain agent indicates future promise for a practical diagnostic assay [158].

Adenovirus

The importance of adenovirus as a cause of diarrheal illness has been difficult to establish, since they have often been visualized in stools from children who do not have enteritis. However, it has recently been established that two of the 41 known adenovirus types, adenovirus type 40 (Ad 40) and adenovirus type 41 (Ad 41), are clearly implicated as etiologic agents of gastroenteritis. They cause 5–10% of diarrhea in children less than three years of age (although the total extent of disease probably has not yet been determined [159], and clinically resemble Rotavirus infection in many respects. In contrast to Rotavirus, Ad 40 or 41 associated diarrhea is typically more persistent, with one-third having diarrhea for as long as 14 days [160], and vomiting is milder. Respiratory symptoms are seen in about one-fifth of patients.

Procedures

Ad 40 or 41 can be visualized by EM during acute stages of the illness when they are excreted in large numbers [161]. Ad 40 and 41 are 70 nm in size and contain DNA in their genome. They cannot be cultivated in cell lines traditionally used for isolation of adenovirus. They can be isolated in a human embryonic kidney cell line (Graham 293) which has been transformed by adenovirus type 5-sheared DNA [145], and these growth characteristics were initially used to identify EAd, but this has since been shown to be unreliable. Ad 40 and Ad 41 are closely related antigenically, but they can be distinguished by a neutralization test in certain cell lines [161].

Several nonculture tests for detection of adenovirus type 40 and 41 have been reported, including polyclonal antibody-based enzyme immunoassay tests, direct genome profiling of virus in stools, nucleic acid hybridization techniques, and quantitative electron microscopy [162]. A monoclonal antibody-based immunoassay has recently been developed and reported to be both sensitive (compared to isolation by growth in Graham 293 cells), and specific as determined by genome profiles after digestion with SmaI endonuclease [162].

Recommendations

Because Ad 40 and 41 cannot be distinguished morphologically from nonpathogens, EM is of no use in diagnosis. The definitive means for isolation and identification of these viruses has been growth in cell culture followed by viral genome typing. Analysis of viral DNA or polypeptides requires large amounts of viruses and is therefore not practical for routine diagnostic work. The immunoassays are rapid, specific, and, presently, the optimal method for detection of the two EAd types. A combined commercial test kit based on specific latex agglutination for detection of Adenovirus and Rotavirus has recently been marketed (Adenolex and Rotalex, Orion Diagnostica, Espoo, Finland) and is reported to be greater than 90% sensitive and 98% specific for both Rotavirus and Ad [163], but is limited in usefullness because of difficulties and distinguishing pathogenic and nonpathogenic strains.

Other Viruses

Calicivirus

Caliciviruses have been reported primarily in children in community-wide and nosocomial outbreaks of gastroenteritis, as well as from sporadic

cases in various parts of the world. There appear to be at least four or five serological types of human calicivirus by IEM, none of which cross-react with known animal caliciviruses. By electron microscopy, caliciviruses have a scalloped border and cup-shaped indentations on their surface that can result in a Star of David appearance. They are 31–35 nm in diameter and contain a single, plus-stranded RNA genome [164]. Rises in serum titers to Norwalk virus have been noted after calicivirus infection, and this in addition to similarities in a structural protein indicate these viruses may be related. Caliciviruses of human origin have only recently been cultivated in vitro [165] and an RIA assay has been developed [166], so this should facilitate additional studies of this agent.

Astrovirus

Astroviruses have been detected in large numbers by IEM during outbreaks of gastroenteritis from stools of young children, in pediatric wards, and in nursing homes. They seem to be of low pathogenicity, since few volunteers orally inoculated with stool filtrates have developed illness even though they shed the organisms in large numbers. At least five serotypes have been reported. They have a characteristic morphology under EM, having an electron dense center with triangular electron-lucent areas resulting in a five- or six-pointed star appearance. Cultivation has been difficult, but serial passage in embryonic human kidney cells and subsequent adaptation to growth in a continuous monkey kidney cell line, LLCMK2, has been reported [167].

Small Round Viruses

Little is known about this poorly defined group of viruses with names such as Otofuke agent, Sapporo agent, minireovirus, and minirotavirus. For the most part, their significance as etiologic agents of gastroenteritis remains to be established. They have been reported from a variety of outbreaks but have also been detected in well individuals. They are generally small (20–30 nm), and they lack a distinct ultrastructure. Little is known about their biophysical or biochemical characteristics. Cultivation and the development of specific assays has not been achieved.

Protozoal Parasites

Diagnosis of intestinal parasitic disease still depends on the direct microscopic evaluation of stool specimens, in adequate numbers, by

experienced observers. To recognize the cyst and trophozoite forms, stools must be appropriately collected, prepared and preserved. Concentrating methods may be used to obtain larger numbers of cyst forms. Advances have been made in tests for host serologic responses, but these are usually more helpful for studies of disease prevalence in population groups than for diagnosis of disease in individual patients. One exception is the use of parasite antigen specific IgM assays which may indicate recent infection. Parasites and their antigens can now be detected and identified in stool with monoclonal immune reagents using fluorescence microscopy, counter-immunoelectrophoresis (CIE) and ELISA. This is true for Giardia and Cryptosporidia. Although axenic culture has been achieved for both amoebae and giardia, culture of protozoa remains a research tool which is not routinely utilized for diagnostic purposes. Culture can be used to permit examination of protozoal enzyme patterns (zymodemes) and DNA restriction digest patterns which may be related to pathogencity. No DNA probes are currently available for parasites. This section will review newer methods for the diagnosis of *Entamoeba histolytica, Giardia lamblia* and *Cryptosporidia.*

E. histolytica

Current estimates of prevalence [168] place *E. histolytica* third among the causes of protozoal-induced deaths (after malaria and schistosomiasis). Amoebiasis may present as invasive enteric disease (Dysentery), as liver abscess or as asymptomatic cyst passage.

Diagnosis is by stool examination. Even in dysenteric cases, the stools contain very few polymorphonuclear leukocytes (fewer than 50/hpf) in contrast to the findings in bacillary dysentery. Examination of rectal biopsies has not proven more efficient than the careful examination of three separately collected stool specimens performed both in fresh saline suspensions and after concentration by the zinc sulfate or formal-ether method [169]. One cannot differentiate the casts of pathogenic and nonpathogenic amoebae, although motile pathogenic trophozoites can be distinguished by their phagocytosis of red cells (hematophagous). The observation of scrapings from active ulcers for trophozoites in fresh saline suspension maintained at 37 °C may improve chances of diagnosis of active disease. Standard polyclonal antisera have been developed to detect *E. histolytica* antigen in stool, but these are no longer commercially available [170].

Serologic tests for serum antibodies are most helpful when there is difficulty in performing the stool exam because of interfering sub-

stances such as barium, bismuth or clay; in cases of invasive disease with liver abscess; and in epidemiologic studies. Antibody can be sought by hemagglutination (HA) inhibition, ELISA or agarose gel diffusion. Stool antibody can be detected by these techniques as well, in particular by counterelectrophoresis (CEP) and indirect immunofluorecence (IF).

An area of recent controversy has been the validity of distinguishing *pathogenic* from *nonpathogenic* forms of *E. histolytica*. Debate has centered around the question of whether presumed nonpathogenic forms are only phenotypic variants or whether they represent genetically distinct strains. If the former case obtains, then there may be no true nonpathogenic *E. histolytica*, since the nonpathogenic phenotypes might revert to virulence in response to as yet undetermined stimuli. Pathogenic and nonpathogenic variants have been described based on their isoenzyme patterns (zymodenes) of the 4 enzymes glucose phosphate isomerase, phosphoglucoconvertase, linolate NADP oxyreductase and hexokinase (two fast bands in pathogens). Twenty-two different zymodenes have been described [171, 172]. The technique involves axenic culture of the isolates [173], lysis, and starch gel electrophoresis followed by visualization of enzyme bands. However, Mirelman et al. [174] have been able to achieve the convincing in vitro conversion of nonpathogenic amoebae to pathogenic pheotypes by manipulating the bacterial flora. Nevertheless, clinical outcome appears to correlate with zymodene pattern. Recently, Strachen et al. [175] reported the use of 2 monoclonal antibodies prepared against amoebal lysates of a strain with pathogenic zymodene, which appear capable of rapidly identifying cultures (but not cysts) of the fast hexokinase phenotype in an immunofluorescence assay. In vivo, one factor necessary for amoebic virulence appears to be the presence of appropriate colonic bacteria. Putative amoebic virulence factors include a lectin which promotes attachment to epithelial cells and is mitogenic for T lymphocytes [176]. A monoclonal antibody to this lectin has been produced but is not yet used for routine diagnosis.

Recommendation

Diagnosis of amoebiasis depends on the skilled examination of stool specimens for cysts and trophozoites by multiple methods, using both fresh samples and stored, concentrated samples. One must recognize the presence of agents which can interfere with the diagnosis by destroying trophozoites. Serology is most useful for invasive disease, but may be

helpful in active disease if stool examinations are inadequate. Methods using ELISA or IFA to detect antigen in stool are not commercially available. Culture methods remain a research tool. The use of zymodene analysis to distinguish pathogenic from nonpathogenic strains is also not generally available and it remains controversial whether the enzyme patterns identify genetically different strains or only reflect phenotypic variation which may be induced by changes in the parasite's environment. The present weight of evidence appears to favor nontreatment of "non-pathogenic" phenotypes.

Giardia lamblia

G. lamblia is the most common pathogenic intestinal protozoa in the US and in underdeveloped areas it is a nearly universal infection of infants. Populations at risk for this water-borne, flagellated protozoan include children in day-care centers, travellers to endemic areas where water supplies are contaminated, backpackers, and immunocompromised hosts including those with IgA deficiency and common variable hypogammaglobulinemia. Water supplies may be contaminated by beavers and muskrats. Age-specific incidence in day care centers indicate the possibility of person to person transmission. The parasite colonizes the uper small bowel, and diagnosis is complicated by the fact the parasites may not be detectable in the stool for 7–14 days after the onset of symptoms as shown in studies of travellers returning from Russia to Finland [177]. Thereafter, stool excretion of cysts may be intermittent. Onset of symptoms is insidious with abdominal bloating and distention accompanying diarrhea. The predilection of the organisms for the upper small bowel appears to be related to a requirement for bile salt for optimal growth conditions. Pathogenesis of the diarrheal disease caused by giardia is not understood. Putative virulence determinants include a surface lectin which may be activated by trypsin and duodenal secretions [178].

Diagnosis is by stool examination, duodenal fluid aspirate and small bowel biopsy. Stool examination for ova and parasites should utilize iodine stained wet smears, trichrome stained cyst concentrates (performed by formalin ethyl acetate centrifugation or zinc sulfate concentration) and trichrome stained polyvinyl alcohol (PVA) preserved stools. Three stools should be examined. Sensitivity is 50–70%. Duodenal fluid samples may be obtained by the Enterotest® which is an absorptive sponge tethered on a

140 cm string. Small bowel biopsy yields a crush or smear preparation for direct microscopic examination but may not be useful early in disease [179]. In the event of high clinical suspicion and negative diagnostic tests a treatment trial with metronidazole may be undertaken.

Immunodiagnostic reagents have been developed for the detection of giardia antigen in stool or intestinal fluid samples. CIE [180] and ELISA [181, 182] have been used by research laboratories with 90% specificity and sensitivity. Alexon biomedical (Rolling Meadow, Ill.) recently marketed a "prospect/giardia" EIA test which utilizes tubes coated with antigiardia monoclonal antibody which are used in a sandwich Elisa. This test is based on the monoclonal antibody to giardia antigen (GSA 65) described by Rosoff and Stibbs [183]. Meridian Diagnostics (Cincinatti, Ohio) has marketed an indirect immunofluorecent detection procedure (Merifluor/giardia) for the detection of giardia cysts in fixed fecal material or in water supplies.

Serodiagnosis

An IgM ELISA has been developed using trophozoites coated in the wells which has good specificity at 1/200 and 1/400 dilutions of serum for active disease and recent infection. Antigen-specific IgM falls in 2–3 weeks after active infection with treatment [184], whereas antigen-specific IgG remains elevated [185].

Axenic culture of *G. lamblia* is now possible. This technique has permitted differentiation among isolates with respect to surface protein labelling, isoenzymes, chromosomal banding and restriction endonuclease digest patterns, as well as growth in culture and drug sensitivity. Recent studies [186] of experimental human infection of volunteers have shown that Giardia isolates have different virulence properties.

Recommendation

A high index of suspicion must be maintained in chronic illness in travellers, children in day care centers and susceptible hosts. Repeated analysis of stools should yield diagnosis. Enterotest exam is probably not more sensitive. The newer monoclonal antibody tests to detect giardia antigen in stool appear to be highly sensitive and specific and may replace direct microscopic observation of fecal specimens. IgM ELISA has a role in detecting active infection. When suspicion is high, but diagonstic test do not confirm the diagnosis, clinical trial of therapy may be instituted.

Cryptosporidia

The high prevalence of chronic cryptosporidial infection and life threatening diarrhea in patients with HIV infection and AIDS has increased awareness of and diagnostic capability for this once seldom recognized protozoa. It is now recognized as a frequent cause of transient (10–24 days) self-limited diarrheal illness in immunocompetent hosts, in children in day care centers, in water-borne outbreaks (where it is frequently found together with giardia), in seasonal outbreaks during warm humid months, and among travellers [187]. Carriage is extremely prevalent in young children in underdeveloped countries, although lower in breast fed infants. This was initially felt to be a genus with different species for each host. However, when animal (calf)-to-human transmission was recognized [188, 189], it was felt to be a single species genus. At present, six species are recognized, of which all human disease is caused by *C. parvum*. There are many reservoir hosts.

The organism has a unique life cycle within the intestine of a single host, in the lumen and at the apical surface of the intestinal epithelium where it is intracellular but extracytoplasmic in a parasitophorous vacuole [187]. Diagnosis was initially made by light or electron microscopic examination of intestinal biopsies, where projecting organisms appear as basophilic vesicles at the surface on H&E. It is now made by recognition of refractile, 2-μm diameter circular oocytes in phase contrast microscopy of concentrated stool specimens obtained by Sheather's sugar flotation technique. These can also be seen by iodine stain of wet mounts, or conveniently sedimented in 10% formalin-preserved stool by Kinyoun's acid fast stains where they stain red, or modified Ziehl-Nielsen stain [190]. Meridian diagnostics (Cincinatti, Ohio) has recently marketed an indirect immunofluorescent detection procedure (merifluor/cryptosporidium) based on a murine monoclonal antibody against an oocyst determinant [191, 192].

The organisms cannot presently be cultured, but complete development can be shown in cultured human fetal lung cells and chicken or porcine kidney cells infected with oocytes [193].

Serology

Campbell and Current [194] demonstrated serum antibody to cryptosporidal antigens by IIF in recovered immunocompetent hosts and AIDS patients. Serum antibody could not be determined in patients with hypogammaglobulinemia. Their assay utilized intestinal tissues from mice

infected with oocysts obtained from calves. This assay permitted determination of antibody to life forms other than oocysts. Titers of 1/40 were sensitive and specific. 95% of those infected have both IgM and IgG responses by ELISA except in AIDS (16% IgM, 90% IgG) [195]. This assay was used for seroepidemiology studies of infection in two latin American populations in Lperu and Venezuela. In these populations 15–20% had serum IgG and IgM indicating recent infection (highest at age 2–3 years) and 64% had detectable IgG indicating endemic infection in these communities [196].

Recommendation

This organism is not confined to patients with immunodeficiency, but should be considered as a cause of transient illness in a range of immunoompetent hosts. Transmission from animals to man is now established. Detection of oocysts in concentrated stool specimens remains the principle means of diagnosis. This can be aided by immunofluorescent antibody techniques. An ELISA technique for quantitative serology has been developed, replacing the original technique which required infected mouse tissue.

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